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TITLE OF THE INVENTION (500 characters max)

Novel Compounds for the Treatment of Sickle Cell Disease

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Respectfully submitted,

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RG-2002-002

COMPOUNDS FOR THE TREATMENT OF SICKLE CELL DISEASE

Field of the invention

This invention relates to compounds that bind to and inhibit the activity of cytochrome b_5 in the physiological re-reduction of auto-oxidized hemoglobin (methemoglobin), *e.g.*, compounds that interfere with the binding of hemoglobin and/or methemoglobin to cytochrome b_5. The invention further relates to pharmaceutical compositions comprising these compounds, and methods of using these pharmaceutical compositions to increase methemoglobin levels in the blood as a treatment for sickle cell disease.

Description of the Related Art

In the United States the occurrence of sickle cell disease, also known as sickle cell anemia, is relatively low, afflicting only about 70,000 Americans (Jones, C. P. **1995**, *Pharmacy Today, 1*, 1-2), predominantly of African descent. Worldwide, however, sickle cell disease afflicts many millions of individuals (Bunn, H. F.; Forget, B. G. *Hemoglobin: Molecular, Genetic and Clinical Aspects*; W. B. Saunders Company: Philadelphia, **1986**. 502-564). Despite the fact that the molecular mechanism of hemoglobin sickling is well understood (Eaton, W. A.; Hofrichter, J. **1990**, *Adv. Protein Chem., 40*, 63-279) and the role of sickling in the pathology of the disease is clear, a rationally based drug therapy is not available to patients despite many attempts to develop such a therapy over the last few decades (Orringer, E. P.; Casella, J. F.; Ataga, K. I.; Koshy, M.; Adams-Graves, P.; Luchtman-Jones. L.; Wun, T.; Watanabe, M.; Shafer, F.; Kutlar, A.; Abboud, M.; Steinberg, M.; Adler, B.; Swerdlow, P.; Terregino, C.; Saccente, S.; Files, B.; Ballas, S.; Brown, R.; Wojtowicz-Praga, S.; Grindel, J. M. **2001**, *JAMA*, 286(17):2099; Abraham, D. J.; Perutz, M. F.; Phillips, S. E. **1983**, *Proc. Natl. Acad. Sci. U S A, 80*, 324-328; Klotz, I. M.; Haney, D. N.; King, L. C. **1981** *Science,213*, 724-731). Although agents that modify hemoglobin allosterism have been identified through site-directed drug design (Abraham, D. J.; Wireko, F. C.; Randad, R. S.; Poyart, C.; Kister, J.; Bohn, B.; Liard, J. F.; Kunert, M. P. **1992**, *Biochemistry, 31*, 9141-9149), all attempts at hemoglobin-directed antisickling agents have been unsuccessful.

The clinical manifestations of sickle cell disease are highly variable (Bunn, H. F.; Forget, B. G. *Hemoglobin: Molecular, Genetic and Clinical Aspects*; W. B. Saunders Company: Philadelphia, 1986. 502-564; Serjeant, G. R. **2001**, *Br. J. Haematol., 112*, 3-18). In young children, the major concern is the incidence of stroke. Chronic hemolytic anemia, impairment of growth and higher susceptibility to infection are common systemic manifestations. Vaso-occlusive crises are the origin of the most severe symptoms, including stroke and cardiac involvement in cases of "chest syndrome". The incidence of three or more vaso-occlusive crises per year is highly correlated with mortality. With current treatment, life expectancy for sickle cell patients is 40 to 50 years.

Until recently, the only approach to the treatment of sickle cell disease was fluids and analgesics, such as morphine, administered upon the occurrence of vaso-occlusive crises. While transfusion therapy is commonly employed in pediatric cases where the risk of stroke is high, there are serious potential problems with long-term transfusion therapy (Serjeant, G. R. **2001**, *Br. J. Haematol., 112*, 3-18). The risk of stroke is predicted by transcranial doppler measurements of blood flow in the brain. Although transfusion therapy is effective in reducing vaso-occlusive crises, such as stroke, there are several drawbacks. Iron overload is a common side effect, and iron chelation therapy employing desferoxamine is a common adjuvant therapy. Long-term transfusion therapy also carries the risk of alloimmunogenic reactions. There is also a risk of disease transmission that has been minimized with recent advances in diagnostic procedures.

In 1995, hydroxyurea (Jones, C. P. **1995** Sickle Cell Therapy so Effective, Trials end early, *Pharmacy Today, 1*, 1-2; Charache, S.; Terrin, M. L.; Moore, R. D.; Dover, G. J.; Barton, F. B.; Eckert, S. V.; McMahon, R. P.; Bonds, D. R. **1995**, *N. Engl. J. Med., 332*, 317-1322; Rodgers, G. P. **1997** *Semin. Hematol., 34*, 2-7.) became available for the treatment of sickle cell disease. Because it was already used in the treatment of certain leukemias, it was rapidly approved for clinical testing and passed through clinical trials faster than any other drug in recent times. In an extensive study involving nearly 300 sickle cell patients, the occurrence of vaso-occlusive crises was reduced to roughly 50% of that observed in the patients involved (Charache, S.; Terrin, M. L.; Moore, R. D.; Dover, G. J.; Barton, F. B.; Eckert, S. V.; McMahon, R. P.; Bonds, D. R. **1995**, *N. Engl. J. Med., 332*, 317-1322.). It is believed that hydroxyurea works by inducing expression of fetal hemoglobin, however, there are a number of controversies concerning the exact mechanism of action, given that benefits appear to begin before the development of significant levels of fetal hemoglobin (Bunn, H. F. **1999**, *Blood, 93*, 1787-1789). Despite the advantages in the use of hydroxyurea, it is not without significant side effects. Hydroxyurea is myelosuppressive and thus patients must be monitored carefully (Rodgers, G. P. **1997** *Semin. Hematol., 34*, 2-7). Hydroxyurea causes chromosomal fragmentation and is teratogenic and mutagenic but does not appear to be carcinogenic. Because of the mutagenicity and the potential carcinogenicity in the long-term, it is not approved for use in children. Rather, it is approved for use in patients who suffer more than three vaso-occlusive crises a year, a clinical pattern strongly correlated with mortality (Castro, O. **1999**, *Br. J. Haematol., 107*, 2-11).

Relatively recently, bone marrow transplantation has been found to be an effective cure for sickle cell disease (Serjeant, G. R. **2001**, *Br. J. Haematol., 112*, 3-18). This treatment was first discovered when a leukemia patient was given a bone marrow transplant and serendipitously was also cured of his sickle cell disease. Several hundred bone marrow transplants have been performed specifically for the purpose of treating sickle cell disease. This approach is only available to about 18% of sickle cell patients because of the requirement of an HLA matched sibling donor. The procedure is costly and carries significant risks. Mortality because of immune responses ranges from 10% to 15% and subsequent alloimmune responses can be problematic. Thus, although, bone marrow transplantation is a very promising true cure for the genetic disorder, it has significant limitations that prevent widespread use.

It is known that methemoglobin, oxyhemoglobin, and carbonmonoxyhemoglobin, effectively inhibit sickling in patients with sickle cell disease (Franklin, I. M.; Rosemeyer, M. A.; Huehns, E. R. **1983**, *Br. J. Haematol., 54*, 579-587). Furthermore, in individuals with congenital deficiencies in cytochrome b_5 methemoglobin levels rise as high as 50% of total hemoglobin and derivatives in the blood, without any adverse clinical manifestations other than mild cyanosis. Clinical trials performed in the1960's demonstrated the efficacy of methemoglobin in the suppression of vaso-occlusive crises but were limited by the rapid re-reduction of methemoglobin by cytochrome b_5 and hence required massive quantities of compounds such as sodium nitrite, benzocaine or para-aminopropriophenone to maintain sufficient levels of methemoglobin (Beutler, E. **1961**, *J. Clin. Invest., 40*, 56-68). Delivery of the appropriate quantities of these compounds was difficult and the prospect of good patient compliance with such a drug regimen was remote.

Thus, sickle cell disease is a serious problem for which no effective solution is available, and a potentially useful approach to the treatment of the disease would be to increase the amount of methemoglobin in patients having sickle cell disease.

Cytochrome b_5 is the terminal electron donor to methemoglobin in the physiological re-reduction of auto-oxidized hemoglobin (Abe, K.; Sugita, Y. **1979**, *Eur. J. Biochem., 101*, 423-428; Gerbaut, L. **1991** *Clin. Chem., 37*, 2117-2120). Hemoglobin auto-oxidizes at approximately 3% per day. The inhibition of cytochrome b_5's action in the re-reduction of methemoglobin to hemoglobin would lead to an increase in methemoglobin levels and a treatment for sickle cell disease.

The structures for hemoglobin and its derivatives have been previously determined (Perutz, M. F. **1989** *TIBS, 14*, 42-44; Bolton, W.; Cox, J. M.; Perutz, M. F. **1968**, *J Mol Biol, 33*, 283-297) and cytochrome b_5 (Mathews, S.; Czerwinski, E. W.; Argos, P. *The X-*

Ray Crystallographic Structure of Calf Liver Cytochrome b_5; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. VII, pp 107-147). Complete NMR assignments for the rat cytochrome b_5 were determined for both equilibrium forms (Guiles, R. D.; Basus, V. J.; Kuntz, I. D.; Waskell, L. **1992**, *Biochemistry, 31*, 11365-11375; Guiles, R. D.; Basus, V. J.; Sarma, S.; Malpure, S.; Fox, K. M.; Kuntz, I. D.; Waskell, L. **1993**, *Biochemistry,. 32*, 8329-8340). In addition, extensive characterization of the structural, dynamic, and electrochemical properties of rat cytochrome b_5 have been performed (Dangi, B.; Sarma, S.; Yan, C.; Banville, D. L.; DiGate, R. J.; Guiles, R. D. **1998**, *Biochemistry,37*, 8289-830251-54; Dangi, B.; Blankman, J. I.; Miller, C. J.; Volkman, B. F.; Guiles, R. D. **1998**, *J. Phys. Chem. B,102*, 8201-8208; Sarma, S.; Banville, D.; DiGate, R. J.; Miller, C.; Guiles, R. D. **1997**, *Biochemistry,36*, 5658-5668; Cheng, J.; Terrettaz, S.; Blankman, J. I.; Miller, C. J.; Dangi, B.; Guiles, R. D. **1997**, *Israel Journal of Chemistry,37*, 259-266; Blankman, J. I.; Shahzad, N.; Dangi, B.; Miller, C. J.; Guiles, R. D. **2000**, *Biochemistry,39*, 14799-14805). Furthermore, theoretical studies of the hemoglobin and cytochrome b_5 complex have been performed (Poulos, T. L.; Mauk, A. G. **1983**, *J. Biol. Chem., 258*, 7369-7373).

Brief Description of the Invention

The present invention is directed to compounds that perform the function described above. The inventive compounds described herein comprise, *e.g.* parts R1, R2, and R3, wherein R1 is a moiety that binds to specific sites on cytochrome b_5 in a way that mimics hemoglobin binding to cytochrome b_5; R2 is a flexible linker that covalently crosslinks R1 and R3; and R3 is a moiety that binds to specific sites on cytochrome b_5 in a way that mimics ATP binding to cytochrome b_5. The inventive compounds further comprise, *e.g.*, parts R1, R2, and R3, wherein: R1 is a moiety that inhibits hemoglobin binding to cytochrome b_5; R2 is a flexible linker that covalently crosslinks R1 and R3; and R3 is a moiety that inhibits electron transfer to methemoglobin by cytochrome b_5. The compounds of the invention further comprise, *e.g.*, parts R1, R2, and R3, wherein R1 is a linear polyamine or a cyclic polyamine such as hexacyclen; R2 is a flexible linker which covalently crosslinks R1 and R3; and R3 is ATP or an ATP derivative.

The present invention is also directed to pharmaceutical compositions of the compounds described above and a method of administering the described compounds to achieve a therapeutic effect in a patient with sickle cell disease.

Brief Description of the Drawings

Figure 1 illustrates sections of contour plots of overlays of $^1H - ^{15}N$ HSQC spectra of cytochrome b_5 by itself and in complex with human methemoglobin. In Figure 1A, the concentration of cytochrome b_5 is 1 mM and the concentration of methemoglobin is 0.50 mM. In Figure 1B, the concentration of methemoglobin is 0.25 mM.
Figure 2 illustrates the heteronuclear correlation spectra (HSQC spectra) of a 2 mM solution of cytochrome b_5 by itself (black contours) and that of a solution containing 2 mM cytochrome b_5 and 4 mM hexacyclen (gray contours).

Figure 4 shows modification of hexacyclen to enable attachment of an R2 linker for use in crosslinking to the R3 moeity.
Figure 5 shows the thiolation of ADP that can then be linked to the derivatized polyamines.
Figure 6 shows linking of derivatized spermine to derivatized ADP.
Figure 7 shows attachment of the flexible spacer and covalent attachment of the two derivatized groups.
Figure 8 shows an HSQC overlay of a sample containing cytochrome b_5 and ATP (2 mM) and a sample of cytochrome b_5 alone.
Figure 9 shows a set of traces of the optical absorbance changes occurring at 577 nm for cytochrome b_5 and methemoglobin at 5 μM concentrations. Various concentrations of buffer and of hexacyclen were examined. In trace A) the buffer concentration is 10 mM phosphate at pH 7.0. In trace B) the buffer concentration is 1 mM phosphate at pH 7.0. In trace C) the

buffer concentration is 1.0 mM phosphate pH 7.0 and the concentration of hexacyclen is 100 μM. In trace D and E) the concentration of phosphate is 1 mM pH 7.0 and the concentration of hexacyclen is 1 mM.

Detailed Description of the Invention

This invention relates, *e.g.*, to compounds having the structure R1-R2-R3 that bind to cytochrome b_5 and inhibit the activity of cytochrome b_5 in the reduction of methemoglobin to hemoglobin. Without wishing to be bound to any particular mechanism, it is proposed that these compounds prevent the binding of hemoglobin and/or methemoglobin to cytochrome b_5 by binding at the methemoglobin/hemoglobin binding site on cytochrome b_5 and preventing the electron transfer between methemoglobin/hemoglobin and cytochrome b_5. By binding to cytochrome b_5 and preventing reduction of autoxidized hemoglobin, these compounds raise the level of methemoglobin in red blood cells and reduce the incidence of cell sickling. Thus, these compounds are useful for the treatment of sickle cell disease.

In one embodiment, the invention relates to a compound that consists of three parts, designated R1, R2 and R3 as described below. R1 and R3 bind to specific sites on the surface of cytochrome b_5 as defined by shifts in ^1H-^{15}N heteronuclear correlation spectrum peaks defined below (Heteronuclear Single Quantum Coherence (HSQC) mapping: Mori, S.; Abeygunawardana, C.; Johnson, M. O. N.; van Zijl, P. C. M. **1995**, *Journal of Magnetic Resonance, Series B, 108*, 94-98). R2 is a linker which covalently links R1 and R3.

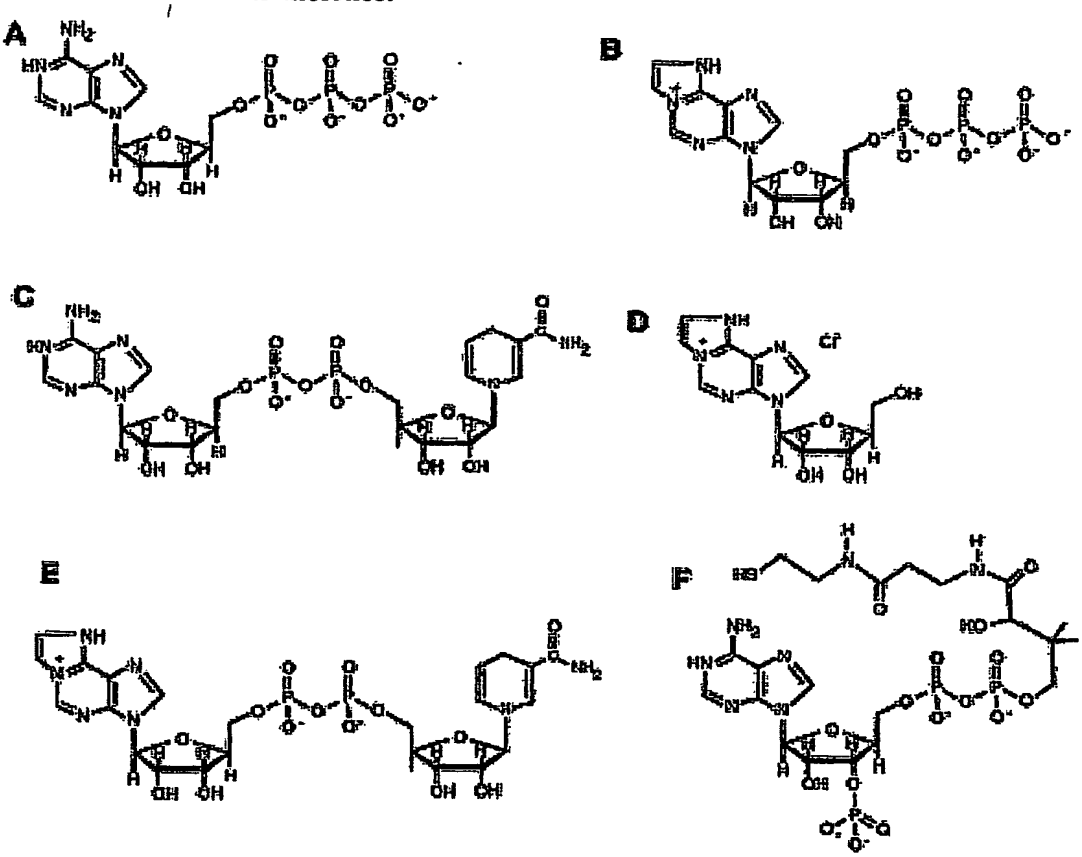
In another embodiment, R1 is 1,4,7,10,13,16-hexaazacyclooctadecane (hexacyclen), the structure of which is as follows:

R1 can be hexacyclen or a derivative thereof that binds to cytochrome b_5 in such a manner as to inhibit electron transfer from cytochrome b_5 to methemoglobin. One can use an optical assay of electron transfer rate to identify such agents (see examples below). It is believed that agents which interfere with electron transfer between cytochrome b_5 and methemoglobin also bind to one or more of the following cytochrome b_5 residues: H26, E43, E44, E48, A54, D60, H80 and A88. Shifts in HSQC perturbation mapping (see examples below). (Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. **1996**, *Science, 274*, 1531-4)

R2 is a linker between R1 and R3. It is preferable that the linker be flexible. A number of linkers, flexible and non-flexible, are known in the art field. By way of example only, R2 can be a polyglycine moiety containing between 1 and 3 glycines and derivatives thereof. As another example, R2 can be polyethylene glycol (PEG) and PEG-like moieties such as, but not limited to, polystyrene-PEG, [2-(2-aminoethoxy)ethoxy] acetic acid, allyloxycarbonyl- [2-(2-aminoethoxy)ethoxy] acetic acid, fluorenyl-methoxycarbonyl-[2-(2-aminoethoxy)ethoxy] acetic acid, ter-butyloxycarbonyl-[2-(2-aminoethoxy)ethoxy] acetic acid, benzyloxycarbonyl-[2-(2-aminoethoxy)ethoxy] acetic acid. Alternatively, R2 can be a straight chain or branched chain of carbon and hydrogen where the number of carbon atoms can be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, or more.

R3 is a moiety that binds to the site on cytochrome b_5 distinct from the binding site of R1. It is preferable that R3 binds to cytochrome b_5 in such a manner as to induce shifts in heteronuclear correlation peaks corresponding to one or more of following residues on cytochrome b_5: I24, L25, H26, and H27. Example of moieties which bind to cytochrome b_5 and induce shifts in heteronuclear correlation peaks at one or more of those specified amino acids is ATP (adenosine 5'-triphosphate); 1,N6-ethenoadenosine 5' triphosphate; β-

nicotinamide adenine dinucleotide (NADH); 1,N6-ethenoadenosine hydrochloride; nicotinamide-1,6-ethenoadenosine; and coenzyme A. Other ATP analog exist and are well known in the art-field. ATP binds to cytochrome b_5 with an affinity of 180 μM. Below are the chemical structures of some R3 moieties:



A) Adenosine 5'-triphosphate (ATP); B) 1,N6-Ethenoadenosine 5' triphosphate; C) β-Nicotinamide adenine dinucleotide (NADH); D) 1,N6-ethenoadenosine hydrochloride; E) Nicotinamide-1,6-ethenoadenosine; F) Coenzyme A. All of the compounds are commercially available.

In another embodiment, the invention relates to a method for achieving a therapeutic effect in an animal having sickle cell disease and in need of treatment thereof, comprising administering an effective amount of the compound R1-R2-R3, where R1 and R3 bind to cytochrome b_5 as discussed above and R2 is a linker a discussed above.

This invention also involves the usage of R1-R2-R3 to prevent the reduction of methemoglobin to hemoglobin such that methemoglobin accumulates in the blood. Such an accumulation of methemoglobin is useful for the prevention of sickling events in animals having sickle cell disease.

Cytochrome b_5 plays an important role in reducing methemoglobin levels, as demonstrated experimentally and indicated in individuals with congenital deficiencies in cytochrome b_5, who have abnormally high levels of methemoglobin in their blood. Compounds of the invention can inhibit the activity of cytochrome b_5 by, *e.g.*, blocking the binding of methemoglobin to cytochrome b_5. Compounds of the invention can also achieve the inhibition of cytochrome b_5 activity by, *e.g.*, blocking electron transfer to methemoglobin. Compounds of the invention that are comprised of two moieties such that each moiety binds to different sites on cytochrome b_5 have an affinity for cytochrome b_5 that is greater than the

affinity of either moiety for its individual site. The compounds of the invention are thus highly effective at inhibiting cytochrome b_5 activity and raising levels of methemoglobin in the blood.

The inventive compounds exhibit, *e.g.*, therapeutic activity in raising levels of methemoglobin in the blood, and are effective in treating sickle cell disease by reducing the amount of cell sickling. In accordance with a preferred embodiment, the present invention includes methods of treating animals suffering from sickle cell disease.

The preferred aspects include pharmaceutical compositions comprising a compound of this invention having the structure R1-R2-R3(wherein R1, R2, and R3 comprise the moieties described above) and a pharmaceutically acceptable carrier; a method of inhibiting the activity of cytochrome b_5 in red blood cells by administering the pharmaceutical compositions to an animal; a method of increasing the levels of methemoglobin in red blood cells by administering the pharmaceutical compositions to an animal; and a method of treating sickle cell disease in a mammal, *e.g.*, a human, by administering the pharmaceutical compositions to the mammal.

The present invention also relates to useful forms of the compounds as disclosed herein, such as pharmaceutically acceptable salts and prodrugs of all the compounds. The compounds of the invention can be administered alone or as an active ingredient of a formulation. Thus, the present invention also includes pharmaceutical compositions of compounds having the structure R1-R2-R3(wherein R1, R2 and R3 comprise the moieties described above) containing, for example, one or more pharmaceutically acceptable carriers.

Numerous standard references are available that describe procedures for preparing various formulations suitable for administering the compounds according to the invention. Examples of potential formulations and preparations are contained, for example, in the Handbook of Pharmaceutical Excipients, American Pharmaceutical Association (current edition); Pharmaceutical Dosage Forms: Tablets (Lieberman, Lachman and Schwartz, editors) current edition, published by Marcel Dekker, Inc., as well as Remington's Pharmaceutical Sciences (Arthur Isol, editor), 1553-1593 (current edition).

In view of the high degree of selective inhibition of cytochrome b_5 activity, the compounds of the present invention can be administered to any animal requiring inhibition of cytochrome b_5 activity. Administration may be accomplished according to animal's needs, for example, by intravenous injection. Various solid oral dosage forms can be used for administering compounds of the invention including such solid forms as tablets, gelcaps, capsules, caplets, granules, lozenges and bulk powders. The compounds of the present invention can be administered alone or combined with various pharmaceutically acceptable carriers, diluents (such as sucrose, mannitol, lactose, starches) and excipients known in the art, including but not limited to suspending agents, solubilizers, buffering agents, binders, disintegrants, preservatives, colorants, flavorants, lubricants and the like. Time-release capsules, tablets and gels are also advantageous in administering the compounds of the present invention.

Various liquid oral dosage forms can also be used for administering compounds of the inventions, including aqueous and non-aqueous solutions, emulsions, suspensions, syrups, and elixirs. Such dosage forms can also contain suitable inert diluents known in the art such as water and suitable excipients known in the art such as preservatives, wetting agents, sweeteners, flavorants, as well as agents for emulsifying and/or suspending the compounds of the invention. The compounds of the present invention may be injected, for example, intravenously, in the form of an isotonic sterile solution. Other preparations are also possible.

The compounds can be administered as the sole active agent or in combination with other pharmaceutical agents, such as other agents that raise levels of hemoglobin variants in the red blood cells in order to prevent cell sickling in animals with sickle cell disease.

The dosages of the compounds of the present invention depend upon a variety of factors including the severity of the symptoms, the age, sex and physical condition of the animal, the route of administration, the frequency of the dosage interval, the particular compound utilized, the efficacy, toxicology profile, pharmacokinetic profile of the compound, and the presence of any deleterious side-effects, among other considerations.

By "effective dose" or "therapeutically effective dose" is meant herein, in reference to the treatment of sickle cell disease, an amount sufficient to bring about one or more of the following results: increase the level of methemoglobin in the blood above about 3%; reduce the

level of pain related to sickle cell disease; or reduce the incidence of sickle cell crises. The compounds of the invention can be administered at dosage levels and in a manner customary for ticlopidine hydrochloride, or other drugs used to treat sickle cell disease. For example, ticlopidine hydrochloride is administered at 250 mg bi-daily (see PDR).

In carrying out the procedures of the present invention it is of course to be understood that reference to particular buffers, media, reagents, cells, culture conditions and the like are not intended to be limiting, but are to be read so as to include all related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system or culture medium for another and still achieve similar, if not identical, results. Those of skill in the art will have sufficient knowledge of such systems and methodologies so as to be able, without undue experimentation, to make such substitutions as will optimally serve their purposes in using the methods and procedures disclosed herein.

Example 1

The interaction of cytochrome b_5 with hemoglobin is explored using HSQC perturbation mapping. Figure 1 contains sections of contour plots of overlays of $^1H - ^{15}N$ HSQC spectra of cytochrome b_5 by itself and in complex with human methemoglobin. Significant shifts in the positions of a number of residues of cytochrome b_5 are observed. In Figure 1A, the concentration of cytochrome b_5 is 1 mM and the concentration of methemoglobin is 0.50 mM. The black contours are of a heteronuclear correlation spectrum of cytochrome b_5 by itself while the gray contours are of a sample containing both cytochrome b_5 and methemoglobin. In Figure 1B, the concentration of methemoglobin is 0.25 mM. The pH in all cases was 6.4 and the temperature was 25 °C. A number of residues that shift significantly on complex formation are labeled (*e.g.* most notably H26, E43, E44, A54, H80 and A88). A number of peaks that do not shift significantly on complex formation are also labeled (i.e. K5 and Y30). Heteronuclear correlation spectra were recorded using the fast HSQC sequence (Mori, S.; Abeygunawardana, C.; Johnson, M. O. N.; van Zijl, P. C. M. **1995**, *J. Mag. Reson. B, 108*, 94-98). The shifts in heteronuclear correlation peaks observed on complex formation are consistent at least in part with the theoretical model of the complex between cytochrome b_5 and methemoglobin. Note that shifts in peaks associated with residues E43, E44 and probably A54 via a relayed effect in helix V of cytochrome b_5 are consistent with the theoretical model. Compounds which interact with cytochrome b_5 at one or more of the following amino acids of cytochrome b_5 may interfere with or prevent the binding of methemoglobin to cytochrome b_5, the amino acids being H26, E43, E44, A54, H80 and A88.

Example 2

Hexacyclen (1,4,7,10,13,16-hexaazacyclooctadecane)(Richman, J. E.; Atkins, T. J. **1974**, *J. Am. Chem. Soc., 96*, 2268-2269) binds to cytochrome b_5 such the HSQC spectra of cytochrome b_5-hexacyclen is similar to the HSQC spectra of cytochrome b_5-hemoglobin. The concentration dependence of hexacyclen-induced heteronuclear correlation peak shifts indicates a dissociation constant of roughly 2 mM. Figure 2 illustrates the heteronuclear correlation spectra (HSQC spectra) of a 2 mM solution of cytochrome b_5 by itself (black contours) and that of a solution containing 2 mM cytochrome b_5 and 4 mM hexacyclen (gray contours). The inset in the upper left hand corner of the figure contains a plot of the hexacyclen dependence of the shifts in the peak to peak separation of aspartate 60 (D60) at concentrations of hexacylen ranging from 0.5 to 8 mM. The inset at the upper right is a model for the interaction of hexacyclen based on the shifts observed in the HSQC perturbation study. Solutions were buffered to a pH of 7.0 with 1 mM phosphate buffer and the spectra were recorded at 40 °C. The insert in the upper left hand corner of the figure contains a plot of the hexacyclen dependence of the shifts in the peak to peak separation of aspartate 60 (D60) at concentrations of hexacylen ranging from 0.5 to 8 mM. The insert at the upper right is a model for the interaction of hexacyclen based on the shifts observed in the HSQC perturbation study.

Example 3

Work was performed on cytochrome b_5 in an attempt to find compounds which inhibit or increase cytochrome b_5's properties, such as the reduction potential and binding affinity to cytochrome b_5's electron transfer partners by relayed effects (Rivera, M.; Wells, M. A.; Walker, F. A. **1994**, *Biochemistry, 33*, 2161-2170; Vergères, G.; Waskell, L. **1995**, *Biochemie, 77*, 604-620; Reid, L. S.; Gray, H. B.; Dalvit, C.; Wright, P. E.; Saltman, P. **1987**, *Biochem., 26*, 7102-7107). ATP binds to cytochrome b_5 with an affinity of 180 μM (Reid, L. S.; Gray, H. B.; Dalvit, C.; Wright, P. E.; Saltman, P. **1987**, *Biochem., 26*, 7102-7107). ATP binds to a hydrophobic domain between the four helix bundle that binds the heme and the β-turn in the β-sheet region on cytochrome b_5, this binding site being distinct from the binding site of hexacyclen.

Example 4

Example 5

Schemes for the derivatization of hexacyclen (R1) have been developed using minor modifications of the Richman-Atkins synthesis(Richman, J. E.; Atkins, T. J. **1974**, *J. Am. Chem. Soc., 96*, 2268-2269). The R group in the scheme shown in Figure 4 was a tosyl group in the original synthesis, but has been replaced with a carbamyl group in this modification, which can be selectively removed using L-selectride (Coop, A.; Rice, K. C. **1998**, *Tet. Lett., 39*, 8933-8934), lithium tri-sec-butylborohydride (Aldrich), under mild conditions which will not remove the Ts group. Figure 4 shows a modified Richman-Atkins synthesis of hexacyclen to enable attachment of a derivatizable R group for use in crosslinking to the R3 moeity. A scheme utilizing this modified hexacyclen in the preparation of a polypeptide that is used to link the thiolated ADP shown in Figure 5 using a bifunctional crosslinking agent is shown in Figure 7.

Example 6

In this scheme to link hexacyclen (R1) with an ADP derivative (R3), described by Hermanson (Hermanson, G. T. *Bioconjugate Techniques*; Academic Press, 1995.785 pp649-655), a water soluble carbodiamide is used to activate the terminal phosphate which can then be thiolated with β-mercaptoethylamine via cystamine following reductive cleavage with Cleland's reagent as shown in Figure 5. As shown in Figure 5, a water soluble carbodiamide is used to activate the terminal phosphate that can then be thiolated with β-mercaptoethylamine via cystamine following reductive cleavage with Cleland's reagent.

Example 7

In this scheme to link hexacyclen (R1) with an ADP derivative (R3), a polypeptide containing a C-terminal lysine and N-termination with the derivatized spermine was prepared using standard solid phase methods (Grant, G. A. *Synthetic Peptides: A User's Guide*; W. H. Freeman and Company: New York, 1992, 382). This polypeptide is then crosslinked to the derivatized ADP shown in Figure 4 using the bifunctional crosslinker BMPS (N-(β-maleimido-propyloxy)succinimide) (McKenzie, J. A.; Raison, R. I.; Rivett, E. E. **1988**, *J. Protein Chem., 7*, 581-592) as shown in Figure 6.

Example 8

A similar scheme for linking a polypeptide with an N-terminal hexacylen derivative to the thiolated ADP is shown in Figure 7. In this scheme a polypeptide with a C-terminal lysine and a variable number of glycines is prepared using standard solid phase synthesis techniques. The N-terminal group here is the derivatized hexacyclen.

Example 10

The binding of the R1 and R3 moieties to cytochrome b_5 is characterized using HSQC perturbation mapping experiments. Figure 2 contains an overlay of heteronuclear correlation

spectra (HSQC spectra) of a 2 mM solution of cytochrome b_5 by itself (black contours) and that of a solution containing 2 mM cytochrome b_5 and 4 mM hexacyclen (gray contours), illustrating shifts due to the binding of hexacyclen to ^{15}N-labeled cytochrome b_5. Figure 2 can be compared with Figure 8, which contains an HSQC overlay of a control on that of a sample containing cytochrome b_5 and ATP. Solutions were buffered to a pH of 7.0 with 1 mM phosphate buffer and the spectra were recorded at 40 °C. Figure 8 contains an HSQC overlay of a control on that of a sample containing cytochrome b_5 and ATP at 2 mM concentration. Although there is some overlap in peaks affected by the binding of ATP with that seen with the binding of hexacyclen, some of these effects are probably relayed.

Example 11

In addition to the site interaction studies using NMR, functional assays of inhibition of electron transfer have been performed using manual mixing experiments, similar to those described by Sugita (Abe, K.; Sugita, Y. **1979**, *Eur. J. Biochem.101*, 423 - 428). The electron transfer reactions were monitored by observing absorbance changes at 577 nm similar to experiments performed by McLendon's group (Qiao, T.; Simmons, J.; Horn, D. A.; Chandler, R.; McLendon, G. **1993**, *97*, 13089-13091). Figure 9 contains a set of traces of the optical absorbance changes occurring at 577 nm for cytochrome b_5 and methemoglobin at 5 μM concentrations with 1 mM phosphate buffer at pH 7.0. Various concentrations of hexacyclen were examined ranging from 100 μM to 1 mM. The concentration of phosphate buffer was also examined in order to assess the effect of ionic strength on the rate of the reaction. In all cases the concentration of cytochrome b_5 and methemoglobin is 5 μM and the temperature was maintained at 37°C. For trace A, the buffer concentration is 10 mM phosphate at pH 7.0. For trace B, the buffer concentration is 1 mM phosphate at pH 7.0. For trace C, the buffer concentration is 1.0 mM phosphate, pH 7.0 and the concentration of hexacyclen is 100 mM. For trace D and trace E, the concentration of phosphate is 1 mM, pH 7.0 and the concentration of hexacyclen is 1 mM.

While the disclosure above describes the invention in detail and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Statement of Invention

1. A compound of the general formula R1-R2-R3, wherein
R1 comprises a moiety that binds to specific sites on cytochrome b_5 such that said binding mimics hemoglobin binding to cytochrome b_5;
R3 comprises a moiety that binds to specific sites on cytochrome b_5 such that said binding mimics ATP binding to cytochrome b_5; and
R2 comprises a linker moiety which links R1 and R3.

2. The compound of claim 1, wherein R1 is a linear polyamine.

3. The compound of claim 1, wherein R1 is a cyclic polyamine.

4. The compound of claim 1, wherein R3 is an ATP derivative.

5. The compound of claim 1, wherein R1 is hexacyclen.

6. The compound of claim 1, wherein R3 is ATP.

7. A pharmaceutical composition, comprising the compound of claim 1 or a pharmaceutically acceptable salt thereof.

8. A method of achieving a therapeutic effect in a animal with sickle cell disease, comprising administering an effective amount of the compound of claim 1.

9. A method of reducing the incidence of red blood cell sickling in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 1.

10. A compound comprising parts R1, R2, and R3, wherein
R1 comprises a moiety that inhibits hemoglobin binding to cytochrome b_5.
R2 comprises a flexible linker which covalently crosslinks R1 and R3.
R3 comprises a moiety that inhibits electron transfer to methemoglobin by cytochrome b_5.

11. .

12. .

13. The compound of claim 10, wherein R3 is an ATP derivative.

14. The compound of claim 10, wherein R1 is hexacyclen.

15. The compound of claim 10, wherein R3 is ATP.

16. A pharmaceutical composition, comprising the compound of claim 10 or a pharmaceutically acceptable salt thereof.

17. A method of achieving a therapeutic effect in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 10.

18. A method of reducing the incidence of red blood cell sickling in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 10.

19. A compound of the general formula R1-R2-R3, wherein

R1 comprises a moiety that binds to cytochrome b_5 at one or more amino acids selected from the group consisting of H26, E43, E44, E48, A54, D60, H80 and A88;
R3 comprises a moiety that binds to cytochrome b_5 distinct from said binding of said R1 to cytochrome b_5; and
R2 comprises a linker moiety which links R1 and R3.

20. A pharmaceutical composition, comprising the compound of claim 19 or a pharmaceutically acceptable salt thereof.

21. A method of achieving a therapeutic effect in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 19.

22. A method of reducing the incidence of red blood cell sickling in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 19.

23. The method of claim 8, claim 9, claim 17, claim 18, claim 21, or claim 22, wherein said animal is human.

Abstract

Compounds have been designed to inhibit the action of cytochrome b_5 in the physiological re-reduction of auto-oxidized hemoglobin (methemoglobin), for the purpose of increasing methemoglobin levels in the blood of patients as a treatment for sickle cell disease. Sickle cell disease afflicts millions worldwide, causing morbidity and mortality, and current drug therapies are inadequate and have serious side effects. Bone marrow transplant is a cure currently available only to a fraction of patients, also with serious side effects. This invention consists of variants of endogenous, non-toxic compounds. Administration of the compounds mimics congenital deficiencies in cytochrome b_5, in which methemoglobin levels rise as high as 50% of total hemoglobin and derivatives in the blood, without adverse clinical manifestations. Methemoglobin inhibits red cell sickling and high levels of methemoglobin in the blood induced by the compounds of this invention prevent the symptoms of sickle cell disease.

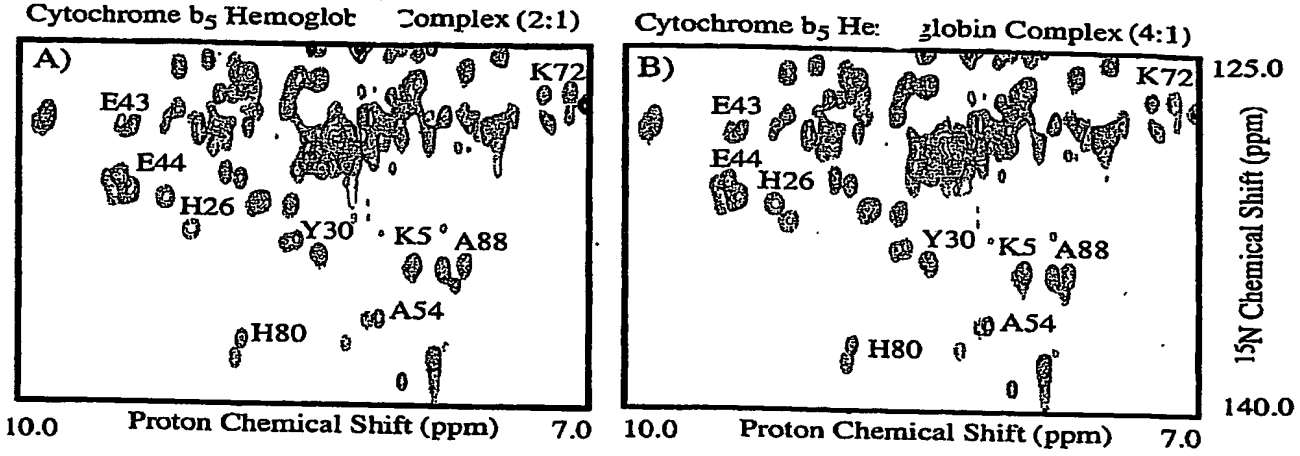


Figure 1

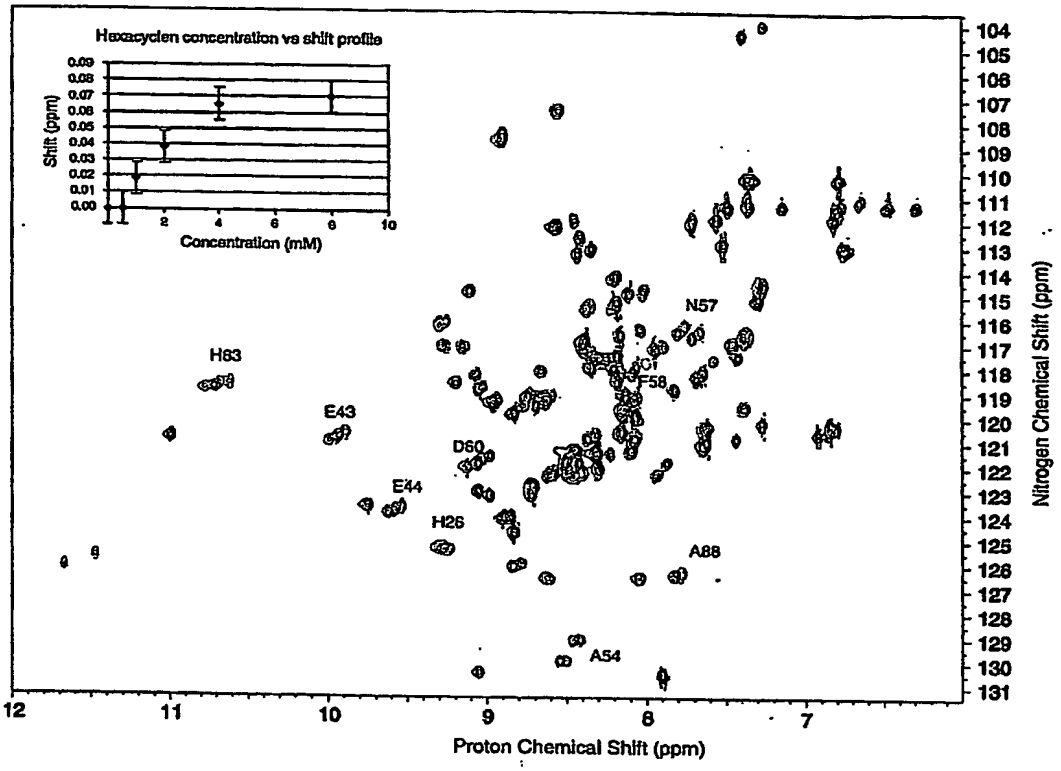


Figure 2

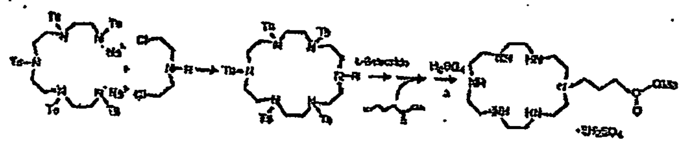


Figure 4

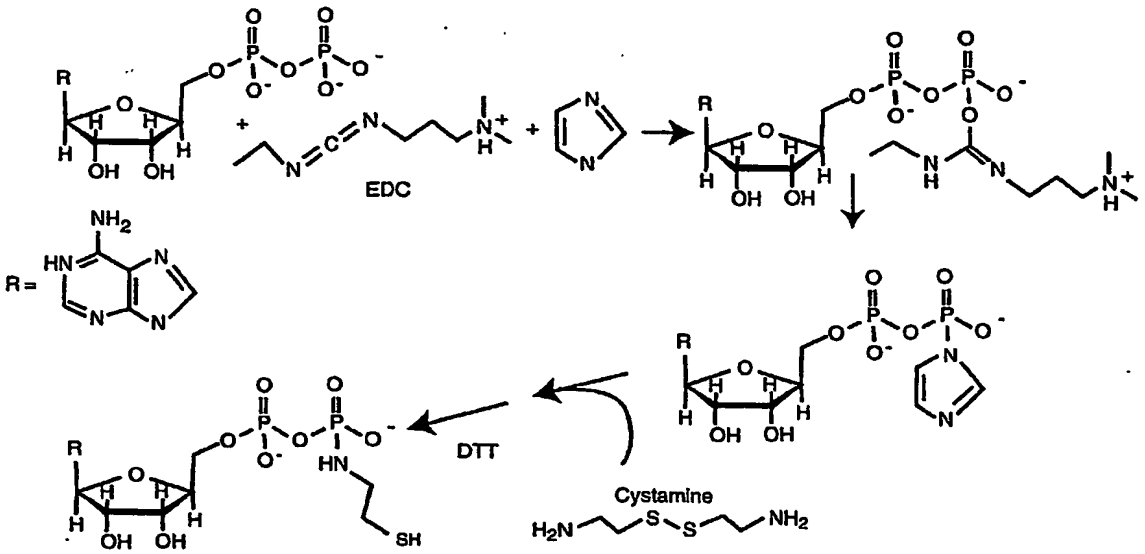
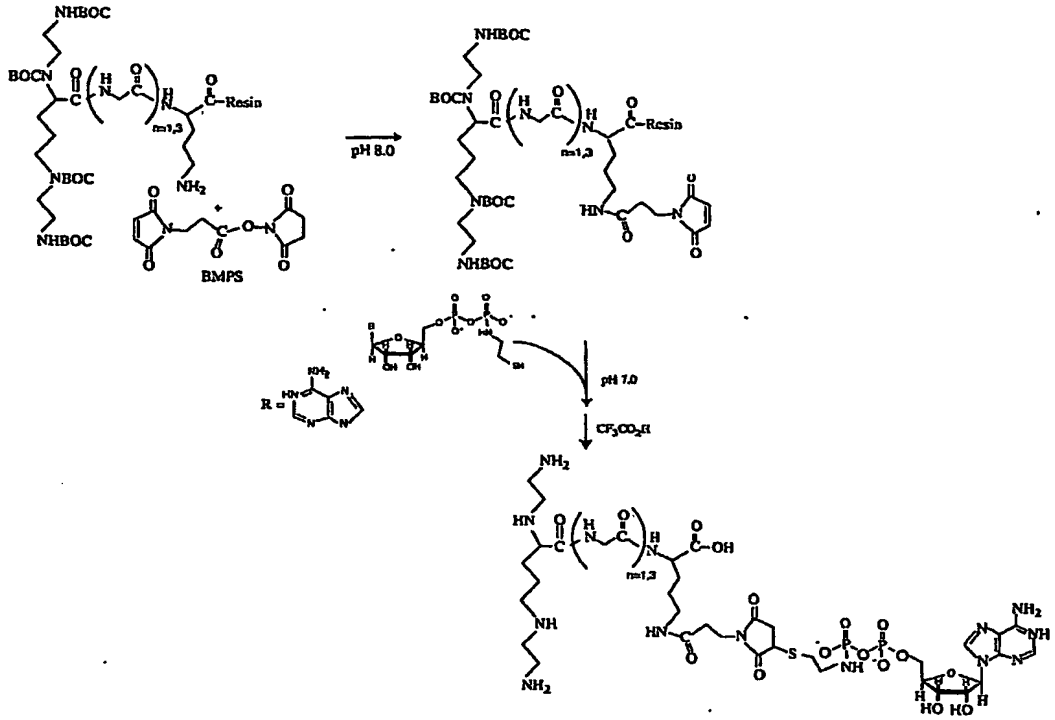


Figure 5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BOCN
NBOC
NHBOC
Resin
m=1,3
NH2
BMPS
pH 8.0
pH 7.0
CF3CO2H
R =
OH
HO HO

Figure 6

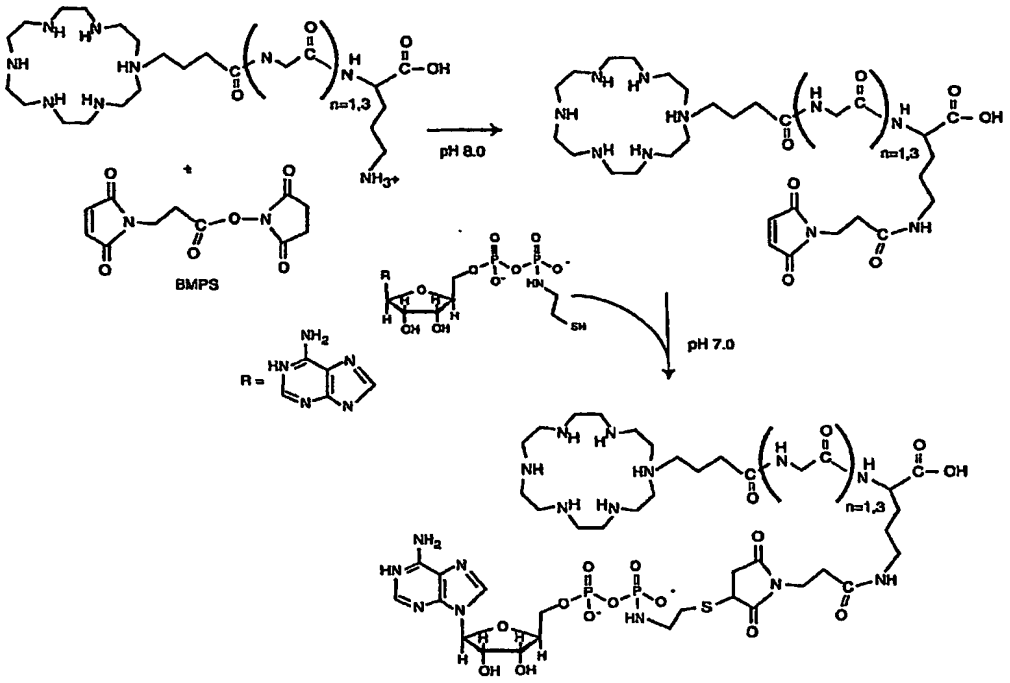
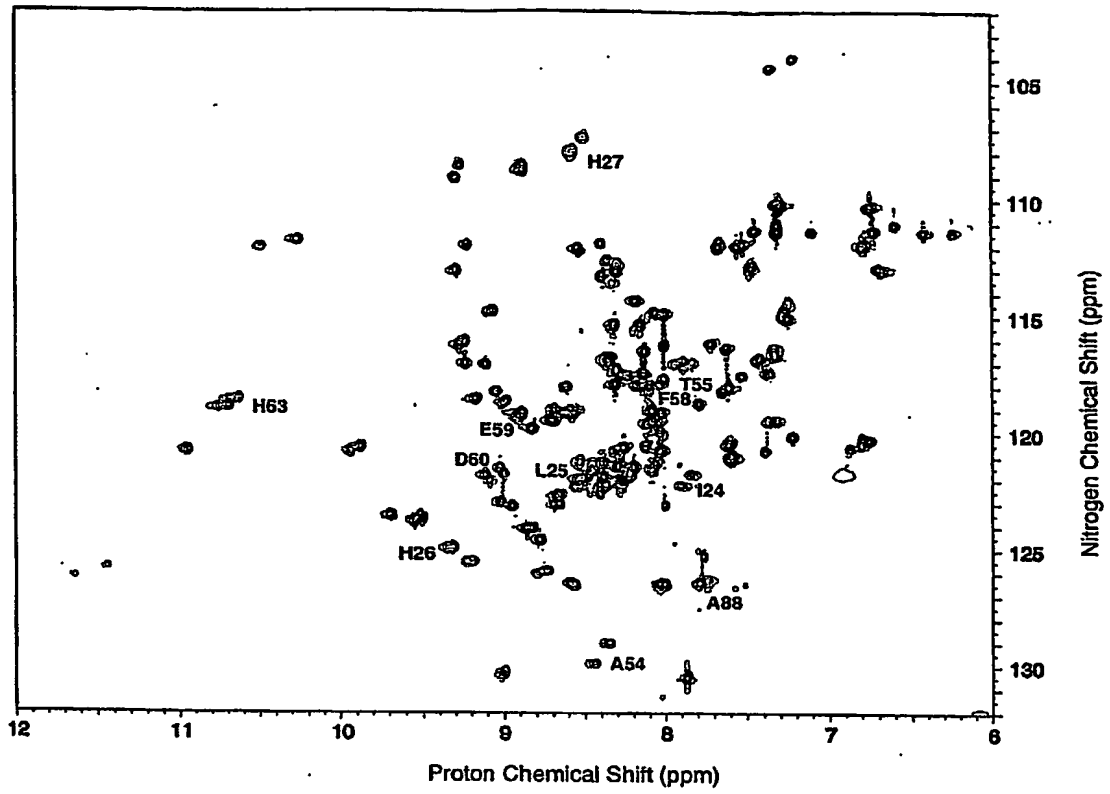


Figure 7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H63
E59
D60
T55
F58
L25
124
H26
A88
A54
105
110
115
120
125
130
12
11
10
9
8
7
6
Proton Chemical Shift (ppm)
Nitrogen Chemical Shift (ppm)

Figure 8

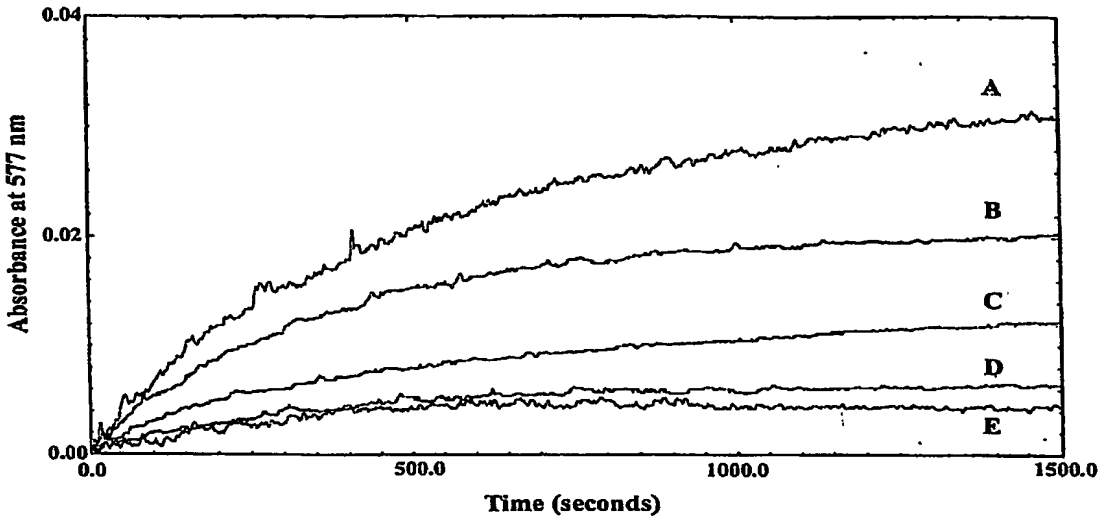
0.04
0.02
0.00
Absorbance at 577 nm
0.0
500.0
1000.0
1500.0
Time (seconds)
A
B
C
D
E

Figure 9